Analysis of Circadian Clock Gene Expression in Human Skin Explants

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Many aspects of skin biochemistry and physiology are known to vary over the course of the 24-hour day. Traditional approaches to study circadian rhythms in the skin have employed rodents or human subjects, which limit the experimental variables that can be studied. Although explants derived from discarded surgical skin are a commonly used model in the skin biology field, circadian rhythms have yet to be examined ex vivo. In this study, using human panniculectomy skin, we used RT-qPCR to monitor the epidermal expression of 4 core circadian clock genes over the course of 1 day ex vivo. Although significant interindividual variability in overall gene expression profiles was observed, robust circadian oscillations were observed in many of the genes and individual explants. Comparison of our gene expression data with microarray data from 2 previous human-subject studies involving primarily young adult White males revealed both similarities and differences, including greater distribution in the time of day of peak expression in the skin explants. This increased variability appears to be due in part to the increased age and altered sex distribution of the donated skin. Nonetheless, our results indicate that skin explants offer an additional experimental system for studying circadian skin biology.

Keywords: Circadian clock, Gene expression, Skin explant, UVR

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INTRODUCTION

Many aspects of physiology exhibit circadian rhythmicity in which gene expression and functional outputs vary over the course of the day. Although the suprachiasmatic nucleus in the brain uses light sensed by retinal ganglion cells in the eye to synchronize circadian rhythms throughout the body (Mohawk et al, 2012), peripheral tissues are capable of autonomously maintaining circadian rhythms even in the absence of light and the suprachiasmatic nucleus. At the molecular level, circadian rhythms are largely governed by a transcription–translation feedback loop in which the CLOCK–BMAL1 complex binds to E-box elements in promoters of target genes to regulate gene expression. Among these clock-controlled genes are period and cryptochrome, which, once translated into protein and localized to the nucleus, feedback and inhibit CLOCK–BMAL1 transcriptional activity (Takahashi, 2017). An additional regulatory loop involves the retinoic acid–related orphan receptor and REV-ERB proteins that competitively bind to related orphan receptor elements in the promoter of the *BMAL1* gene to regulate its expression (Akashi and Takumi, 2005; Guillaumond et al, 2005).

The impact of the circadian clock is pervasive throughout the body, including in the skin (Duan et al, 2021; Lubov et al, 2021; Plikus et al, 2015), where fundamental functions of skin cells as well as responses to external stressors such as viruses and UVR are known to exhibit circadian rhythmicity. However, much of our knowledge of circadian rhythms in skin cells is derived from studies with cultured cells in vitro or from experimental work using rodents and human subjects. Although these approaches are valuable, each experimental system has its limitations. For example, to observe rhythmic gene expression in vitro, cell cultures need to be artificially synchronized with glucocorticoids or other compounds (Balsalobre et al, 2000, 1998) that do not fully mimic in vivo conditions, and even under these experimental conditions, only a very small number of genes exhibit 24-hour periodicity (Hughes et al, 2009). Rodents, including both mice and rats, have been widely used for circadian studies, including in the skin. However, the nocturnal and diurnal nature of rodents and humans, respectively, coupled with structural and physiological differences that exist in the skin of these different organisms may limit the translation of results from rodents to humans. Although there have been significant advances in understanding the molecular make-up of the skin clock through analyses of skin biopsies obtained from human subjects at different times of the day (Del Olmo et al, 2022; Spörl et al, 2012; Wu et al, 2018), the limited amount of tissue that can typically be obtained from a single individual subject restricts the experimental variables that can be examined at 1 time.

Discarded skin from routine surgical procedures has historically provided the skin biology and experimental dermatology fields with an additional and powerful model system for studying various diverse aspects of skin physiology

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(Cousin et al, 2023; Eberlin et al, 2020). Although explants from human colonic (Camello-Almaraz et al, 2020), gastrointestinal (Lago-Sampedro et al, 2021), and brown adipose (Lee et al, 2016) tissues have all been used to study various aspects of circadian biology ex vivo, human skin explants have not previously been explored for circadian studies. In this study, we therefore used discarded surgical skin from primarily middle-aged male and female donors to monitor the expression of core circadian genes at the mRNA level and explore the potential utility of skin explants for circadian analyses.

RESULTS

Human skin explants exhibit interindividual variability in circadian clock gene rhythmicity

To begin to explore whether circadian clock gene expression is maintained ex vivo, we collected panniculectomy skin from a panel of 13 different donors. As shown in Table 1, the donated samples were from individuals ranging from ages 31 to 57 years (average of 45 ± 8 years). Although most (9 of 12, 75%) samples were from females, we obtained 3 (3 of 12, 25%) skin donations from males for our studies. The skin samples were collected at various times from a local hospital between the hours of 10:30 AM and 4:30 PM and brought to the laboratory for ex vivo study. Sections of skin were cut and incubated in a minimal amount of basal DMEM in 10-cm plates that were incubated in a 37 °C water bath throughout the study. Beginning at 6 PM, 8-mm biopsies were taken from the skin and stored in RNAlater at −20 °C, and this process was repeated at 6-hour intervals at 12 AM, 6 AM, and 12 PM. To analyze the expression of circadian clock genes, RNA was purified from the epidermis of the skin biopsy and then subjected to reverse transcription and TaqMan-based qPCR. Relative gene expression was determined by normalization to β-microglobulin and then to the time point with the maximal expression.

The gene expression results for 4 representative circadian clock genes (*ARNTL*, *NR1D2*, *CRY1*, and *PER2*) were then analyzed for each individual skin sample. *ARNTL*, *NR1D2*, and *PER2* were selected because these genes were previously examined and highlighted in an earlier human-subject study (Wu et al, 2018). *CRY1* was included as an additional clock repressor gene. Primary data (C_t values) are provided in Table 1, and data normalized to the time of day of peak expression for each individual skin sample are shown in Figure 1a. For each of the samples, the expression of all 4 core circadian clock genes was found to change over the time frame of the study. However, significant interindividual variability in the expression of these genes was noted among the skin samples. For some skin samples (samples 1, 2, and 5), expression of all 4 of the clock genes decreased between the first 2 time periods (6 PM to 12 AM) but then subsequently recovered. Nonetheless, this observation was not consistently seen in the other samples, such that in most other samples, either increases and/or decreases were observed over the time period depending on the specific gene.

If a gene's expression level or other physiological parameter exhibits circadian rhythmicity, then its data points should

Table 1. Sex and Age of Skin Donors for Human Skin Explant Studies and Circadian Gene Expression RT-qPCR C_t Values

			Skin Sample												
	Gene	Time	1 31/F	2 51/F	3 43/M	4 49/F	5 (?)	6 42/F	7 57/F	8 43/F	9 47/M	10 50/F	11 34/M	12 35/F	13 55/F
C_t	*ARNTL*	6 PM	33.91	33.39	30.86	35.27	32.25	33.13	35.77	36.49	34.26	38.32	33.73	32.76	33.33
		12 AM	34.27	32.32	31.82	33.03	34.20	34.18	40.21	35.52	35.64	37.49	35.12	32.21	33.75
		6 AM	33.31	32.64	30.67	36.05	34.52	35.54	38.00	37.26	36.80	36.99	33.96	33.19	35.16
		12 PM	35.82	33.23	31.97	34.14	33.31	34.65	36.11	35.92	38.42	38.54	35.47	32.90	34.32
	NR1D2	6 PM	32.55	31.35	31.42	32.56	31.15	32.51	33.75	36.39	32.75	35.32	31.30	30.93	32.50
		12 AM	32.23	30.51	33.35	33.14	34.21	33.05	36.08	34.28	34.89	35.96	33.37	30.25	32.16
		6 AM	31.58	31.23	31.78	34.72	33.08	33.26	36.22	36.16	35.77	35.26	31.92	30.85	32.76
		12 PM	32.93	31.37	30.34	31.19	32.16	32.43	33.31	35.57	37.83	37.26	31.91	30.60	32.42
	CRY1	6 PM	32.02	30.42	29.17	32.18	29.28	32.51	33.46	35.06	33.97	36.31	33.41	33.10	34.50
		12 AM	31.98	30.25	30.61	31.75	30.97	31.77	36.82	34.60	37.15	35.85	36.17	33.41	34.08
		6 AM	31.73	29.92	28.59	32.12	30.76	33.14	37.65	35.36	35.69	35.53	33.66	34.87	34.17
		12 PM	34.48	30.44	30.53	31.72	30.87	32.43	33.50	34.82	37.09	36.71	35.33	33.63	32.54
	PER2	6 PM	31.89	30.11	29.52	33.13	29.11	32.09	32.60	36.80	31.59	35.61	31.76	31.67	34.71
		12 AM	31.88	30.00	30.54	33.45	32.00	31.18	36.78	37.48	35.39	37.29	35.56	30.05	32.33
		6 AM	31.73	30.21	28.47	34.40	32.24	32.49	37.24	36.02	33.39	37.27	33.02	32.96	32.46
		12 PM	33.85	31.02	30.52	31.53	31.07	32.35	33.62	37.59	35.91	37.04	33.42	32.32	30.52
	B2M	6 PM	23.31	22.58	22.21	24.02	22.70	24.39	28.86	31.54	27.38	30.71	26.21	25.44	27.14
		12 AM	22.37	21.35	23.59	25.17	22.73	24.43	31.73	29.95	29.00	30.58	28.38	25.13	27.40
		6 AM	22.49	22.04	22.43	25.20	23.19	25.72	31.62	31.91	29.13	30.33	26.97	26.13	27.76
		12 PM	24.93	23.16	23.29	24.34	23.07	24.72	28.87	30.70	30.81	31.77	27.58	25.60	27.09

Abbreviations: F, female; M, male.

The sex and age for each of the 13 skin samples are provided (except for sample 5, which was unknown) along with RT-qPCR C_t values for the indicated genes and time points.

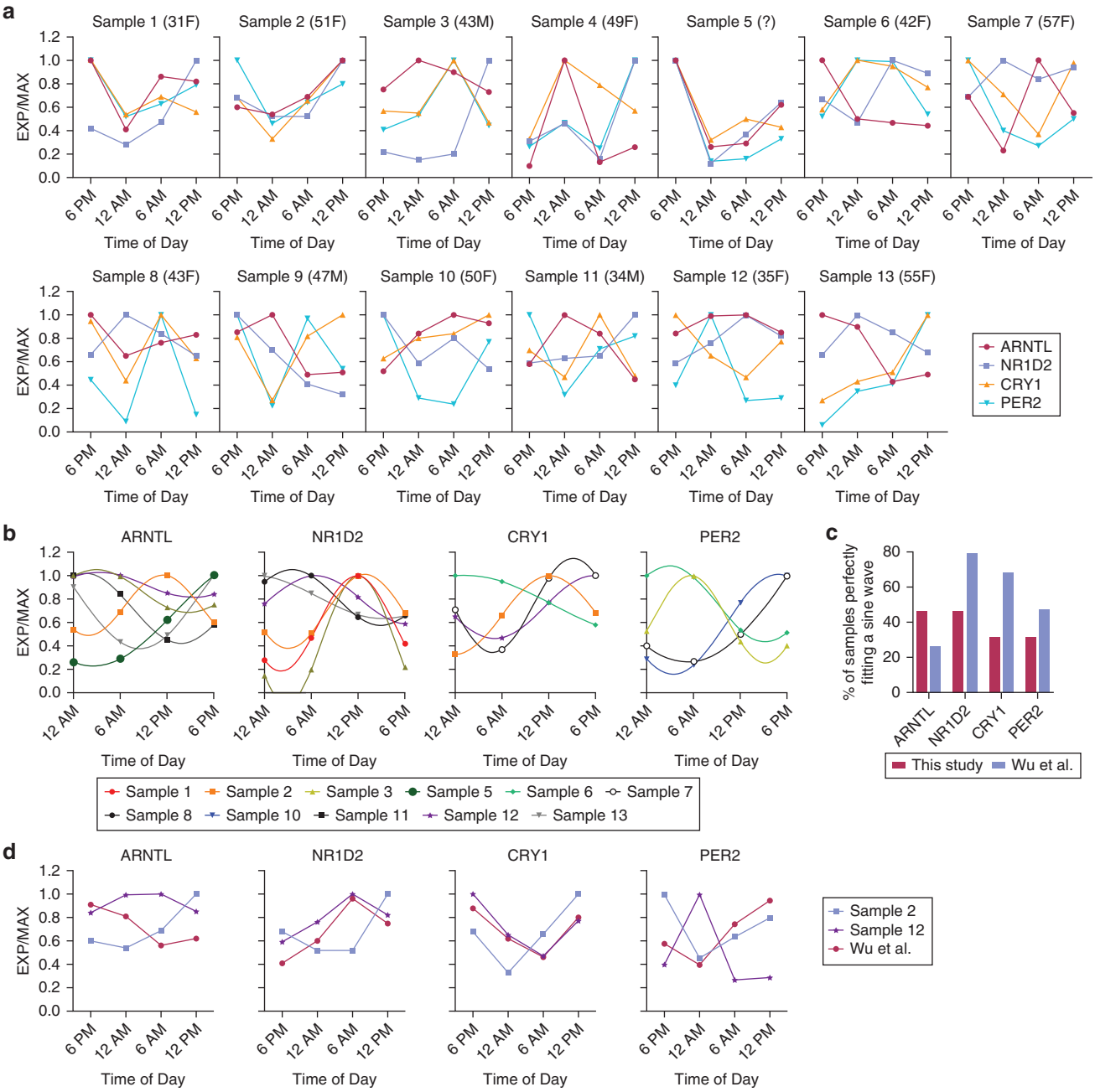

Figure 1. Analysis of circadian clock gene expression in human skin explants reveals significant interindividual variability. **(a)** The expression of the indicated circadian clock gene over the course of the day was analyzed by RT-qPCR from 13 different skin samples. For each skin sample, gene expression was compared with the time point with maximum expression for that gene (EXP/MAX). **(b)** A nonlinear regression analysis was performed using GraphPad Prism to identify 9 instances among the different samples and genes that fit perfectly to a sine wave (with a wavelength of at least 18 hours). **(c)** Comparison of the percentage of samples for each gene in Wu et al (2018) and our study that perfectly fit a sine wave. **(d)** Comparison of circadian gene expression in samples 2 and 12 and in the averaged data in Wu et al (2018). CRY, cryptochrome; EXP, experimental time point; F, female; M, male; MAX, maximum time point.

fit a sine wave. We therefore used GraphPad Prism to perform a nonlinear regression analysis and determine whether any of the gene expression patterns in our skin samples fit a sine wave with a wavelength of at least 18 hours. As shown in Figure 1b, we found that the gene expression patterns perfectly fit a sine wave in 4–6 samples for each of the 4 genes (20 of the total 52 sample gene expression patterns or 38%). This finding was only modestly lower than that observed in a human-subject study that obtained biopsies from forearms of heathy males over the course of the day (42 of 76 samples or 55%) (Wu et al, 2018) (Figure 1c).

Interestingly, in explant samples 2 and 12 of our study, 3 of the 4 circadian genes perfectly fit a sine wave function with a wavelength between 19 and 25 hours. When we compared the expression of the 4 clock genes with the averaged expression from 19 human subjects in the Wu et al (2018)'s study, we found that 2 genes (*NR1D2* and *CRY1*) were nearly perfectly aligned with the expression data in sample 12 and

possibly phase shifted in sample 2. In contrast, the expression of *ARNTL* and *PER2* in the averaged Wu et al (2018) data exhibited differences in the pattern of expression over the course of the day in sample 12 but similarities for *PER2* in sample 2. Thus, in some cases, circadian gene expression in skin explants accurately mimics what occurs in skin in vivo. However, for many genes and skin samples that exhibit rhythmic expression, the pattern is altered or shifted in these explant samples relative to what has been reported in skin obtained from primarily healthy young adult male donors in vivo.

Comparison of gene expression in human skin ex vivo with that in human skin in vivo

There have been 2 microarray-based studies that have monitored gene expression over the course of the day in human skin epidermis (Del Olmo et al, 2022; Wu et al, 2018). To better understand the extent to which circadian clock gene expression in skin explants recapitulates what is found in human skin in vivo, we downloaded the transcriptomic data for these 4 genes from the Gene Expression Omnibus database and then plotted the average relative expression data (Figure 2a). This comparison revealed significant qualitative similarity between the Del Olmo et al (2022) and Wu et al (2018) datasets in terms of changes in expression over the course of the day and the timing of peak and trough expression. In contrast, our averaged data from 13 different individuals did not display any significant oscillations. Moreover, as expected from the results shown in Figure 1a, the variance in gene expression (plotted for each time point throughout the day) was significantly higher among the 4 clock genes in our study than in Wu et al (2018) for all genes except *CRY1* (Figure 2b). Although the variance in our study appeared to trend higher than that in Del Olmo et al (2022), the variance values were not statistically different. Nonetheless, these results indicate that there is more variation in relative circadian clock gene expression in skin explants than in skin in vivo.

When we calculated the amplitude of the gene expression rhythm for each of the subjects in all 3 studies, we observed both similarities and differences with those of Del Olmo et al (2022) and Wu et al (2018) (Figure 2c). Nonetheless, except for *ARNTL* and *PER2*, lower amplitude rhythms were observed in both our study and Del Olmo et al (2022) than in Wu et al (2018). Although lower amplitude rhythms in core clock genes in the Del Olmo et al (2022) dataset than in the Wu et al (2018) dataset was recently reported (Cvammen et al, 2024), the reason for this difference remains to be explored. We note that Wu et al (2018) obtained biopsies from forearm skin, whereas lower buttock skin was used in Del Olmo et al (2022), and abdominal skin was used in our study. Lower buttock and abdominal skin may therefore be more similar to one another than to forearm skin (Cvammen et al, 2024), which could be due to factors such as light exposure or body temperature. Nonetheless, the similarities that exist among these 3 studies suggest that abdominal skin explants may recapitulate certain skin locations in vivo.

Finally, we also determined the time point at which each circadian gene is expressed at its highest level, which is termed the acrophase. Because Del Olmo et al (2022) collected biopsies on a time interval different from that of Wu et al (2018) and ours (4 hours vs 6 hours), we grouped and redefined their 4 AM and 8 AM time points as 6 AM and their 4 PM and 8 PM time points as 6 PM to facilitate this direct analysis. Although the acrophases were similar for all 4 genes in Del Olmo et al (2022) and Wu et al (2018), they were generally more distributed in our study (Figure 2d). This difference may suggest that there is greater variability in 1 or more factors that impacts circadian rhythmicity in the skin explant donors than in the individuals who participated in the 2 other human-subject studies. This could be due to several possible factors, including demographics of the skin donors, chronotype or sleep schedule, or changes to their feeding and behavior prior to surgery.

Circadian gene expression in human skin explants is impacted by age and sex

Because there are differences in the distribution of ages and sexes between our study (ages 31−57 years; 25% male, 75% female) and both Wu et al (2018) (ages 21−49 years; 100% male) and Del Olmo et al (2022) (ages 20−30 years; 45% male, 55% female), we next reanalyzed our clock gene expression data on the basis of age and sex. For age, we separated the samples into 2 groups depending on whether the skin was from a donor aged above or below the mean age of 45 years and then plotted the average gene expression of the 2 age groups over the course of the incubation period (Figure 3a). As shown in Figure 3b, this analysis showed stronger amplitude rhythms for the circadian repressor gene *CRY1* and a trend toward higher expression for *ARNTL* ($P = .06$) in skin explants from individuals aged >45 years than in samples from donors aged <45 years. Similar extents of change were seen for *NR1D2* and *PER2*. Furthermore, similar levels of variation in gene expression were observed at each time point in samples from both age groups except for *ARNTL*, which was higher in older donors than in young donors (Figure 3c), which suggests that skin explants from both age groups can be used to monitor changes in circadian gene expression. Finally, although the numbers of samples were small after grouping on the basis of age, we also noted some differences in the time of peak expression between the 2 age groups (Figure 3d). For example, 5 of the 6 explant samples from individuals aged <45 years showed peak *ARNTL* expression in the evening hours (6 PM and 12 AM), which was analogous to the results in Del Olmo et al (2022) and Wu et al (2018) (Figure 2d) that similarly used human subjects primarily in this age range.

We then performed similar analyses by separating the data on the basis of the sex of the skin donor (Figure 4a). No significant differences were observed in the amplitudes of the gene expression rhythms (Figure 4b). In contrast, analyses of gene expression variance revealed less variation for *ARNTL* and *PER2* in males than in females (Figure 4c). For *ARNTL*, this was likely because in all 3 male skin explants (samples 3, 9, and 11), the peak expression of *ARNTL* occurred at the same time of day (12 AM) (Figure 4a and d). In general, the acrophases were more distributed and variable in females than in males (Figure 4d). However, given the different numbers of samples used in this analysis, which included

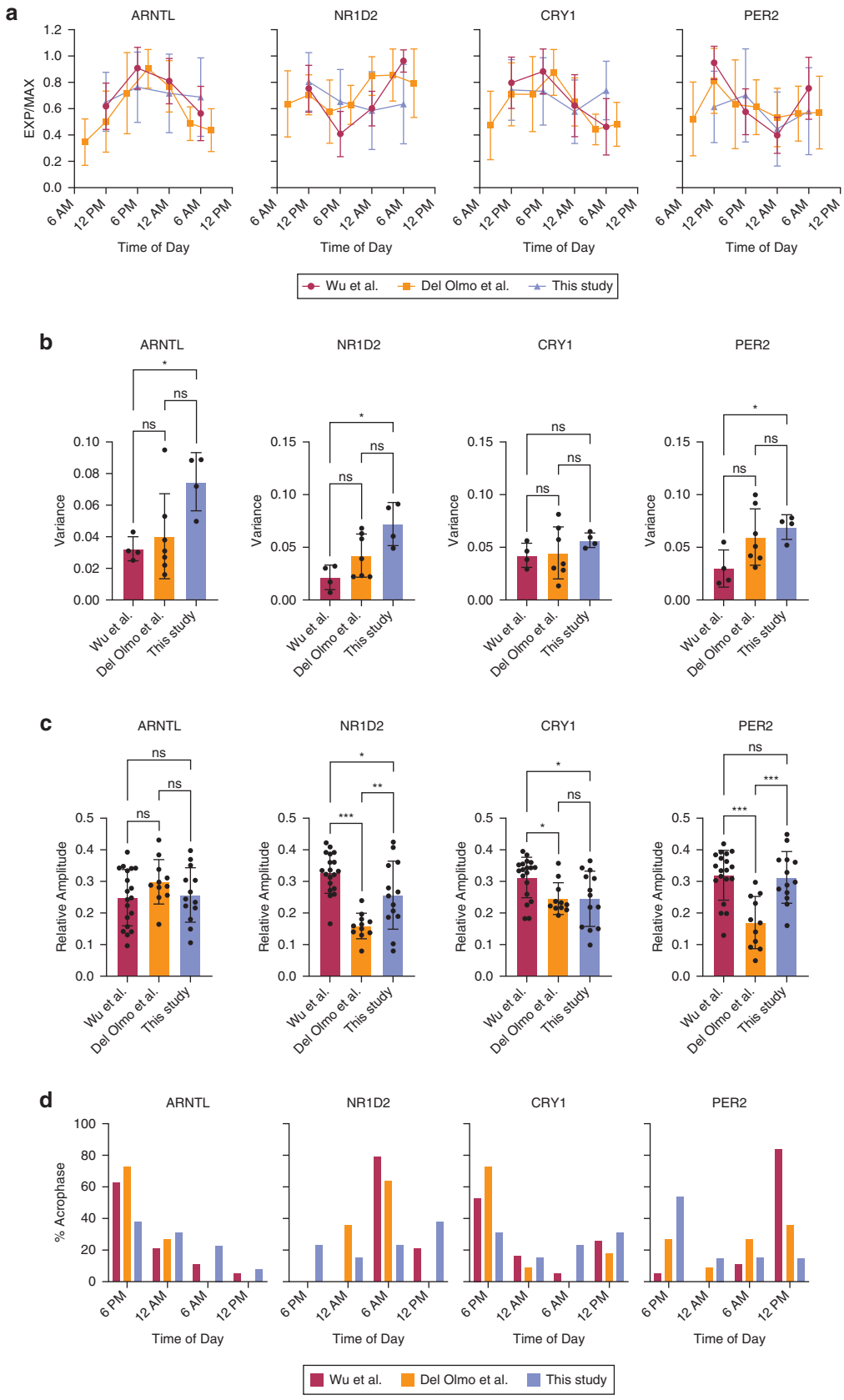

Figure 2. Comparison of gene expression in human skin ex vivo with the expression in human skin in vivo. (**a**) Relative mean gene expression for the 4 circadian genes was averaged from our study (n = 13) and from 2 human-subject studies previously performed by Wu et al (2018) (n = 19) and Del Olmo et al (2022) (n = 11). The error bars show SD of the mean. (**b**) Variance in relative gene expression among the different samples/subjects at each of the time points in the 3 indicated studies (n = 4–6 time points, depending on the study). Each data point represents the average variance at a specific time point of the day, and the bar graph represents the average and SD. (**c**) The amplitude of each individual rhythm was calculated for each subject or skin sample for each of the 4 genes from the 3 studies, and the bar graph shows average and SD. Ordinary 1-way ANOVAs were used to compare the amplitudes and variances of each sample or time point for each the 4 genes in **c** and **d** (*$P < .05$, **$P < .01$, and ***$P < .001$). (**d**) The percentage of samples/subjects with a peak expression at the indicated time point was calculated. CRY, cryptochrome; EXP, experimental time point; MAX, maximum time point; ns, not significant.

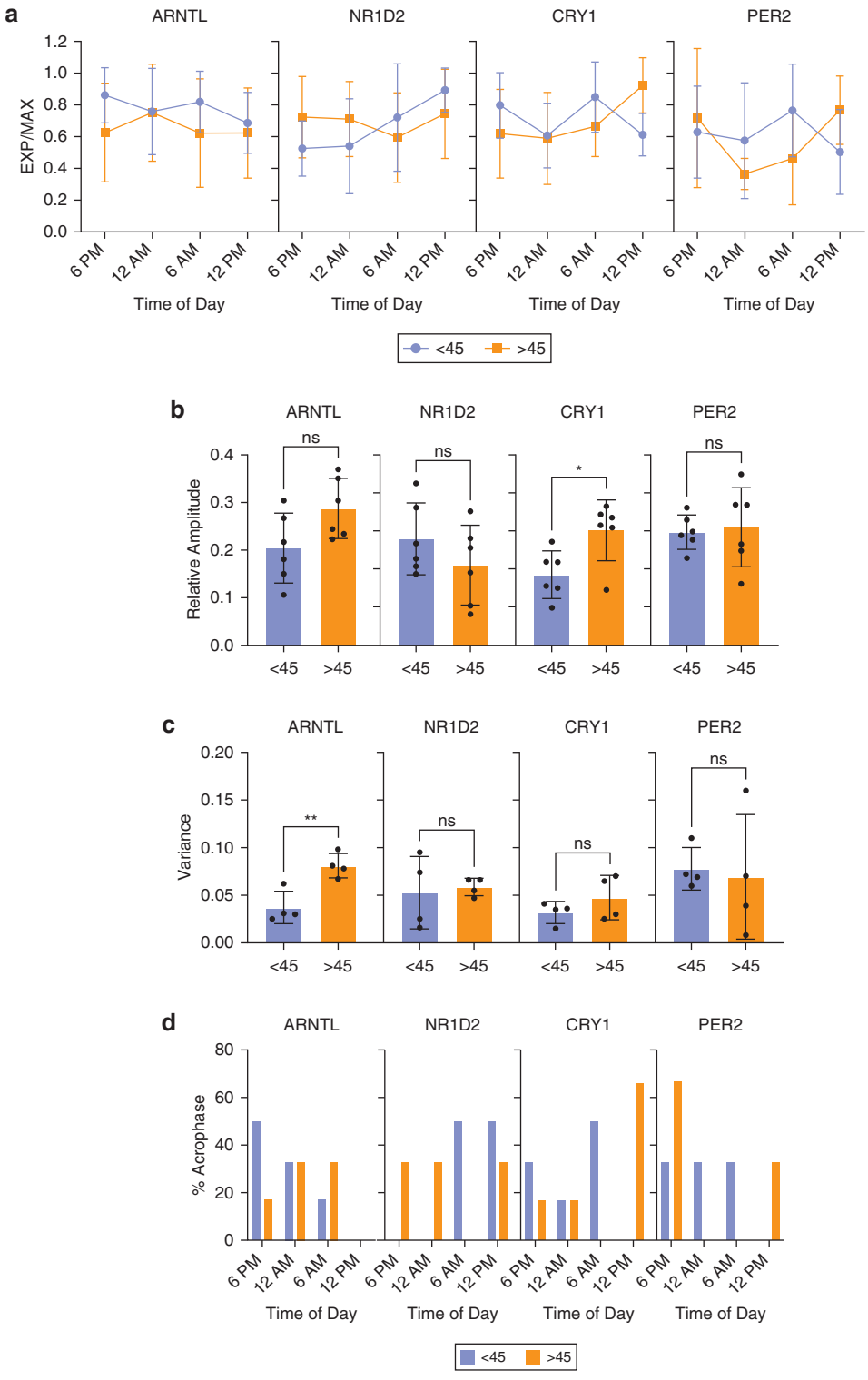

Figure 3. Analysis of circadian clock gene expression in skin explants as a function of age. (**a**) The relative expression of each gene (average and SD) was compared between samples from donors aged ≤45 years (n = 6 for each age group). (**b**) The amplitude of clock gene expression was compared between samples from the 2 age groups. The bar graphs show the average and SD, which were compared with an unpaired *t*-test. (**c**) Variance in relative gene expression was calculated for each time point and gene in the 2 age groups, and the average variance for each time point was graphed along with the SD. An unpaired *t*-test was used to compare the variances in the 2 groups. (**d**) The percentage of samples with a peak expression at the indicated time point was calculated as a function of age group (**$P < .01$). CRY, cryptochrome; EXP, experimental time point; MAX, maximum time point; ns, not significant.

only 3 males, it is possible that future analyses of a larger number of samples may alter this conclusion.

DISCUSSION

The data presented in this paper examined whether circadian oscillations in core clock gene expression can be observed and maintained in human skin epidermis ex vivo. Only a limited number of core circadian genes were analyzed in this pilot study, which may limit the conclusions that can be drawn with respect to the entire clock and known clock-controlled processes. However, although significant interindividual variability in expression at specific time points and expression patterns over time were observed in our work, this issue is likely not unique to skin explant culture. Indeed, interindividual variability in circadian clock gene expression was previously noted in skin biopsies from forearms of

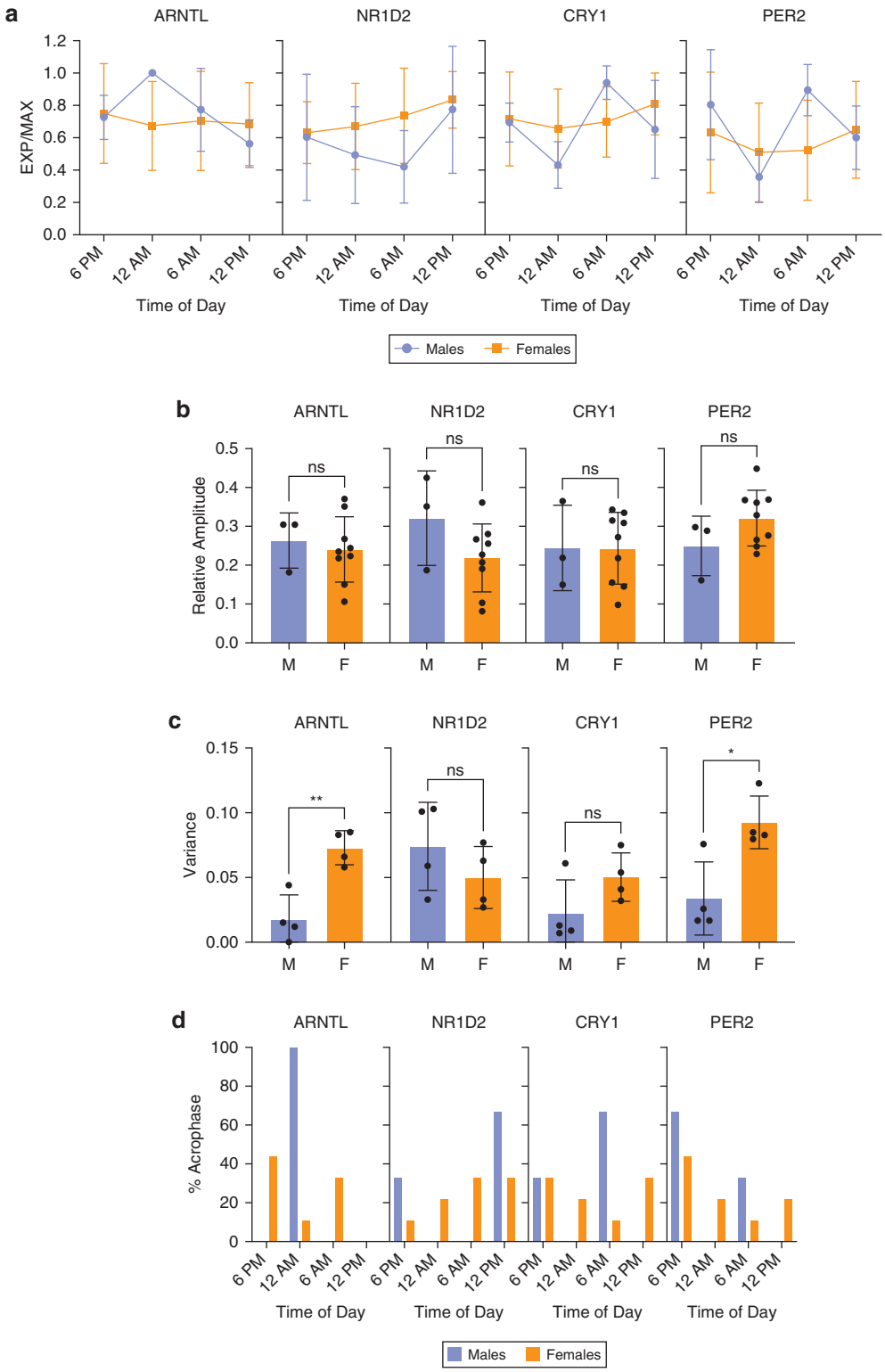

Figure 4. Analysis of circadian clock gene expression in skin explants as a function of sex. (**a**) The relative expression of each gene (average and SD) was compared between samples from male (denoted as M) (n = 3) or female (denoted as F) (n = 9) donors. (**b**) The relative amplitude of clock gene expression was compared between samples from male and female donors. The bar graph shows the average and SD, which were compared with an unpaired *t*-test. (**c**) Variance in relative gene expression was calculated for each time point and gene in the 2 sex groups. The average and SD were graphed and then compared using an unpaired *t*-test. (**d**) The percentage of samples with a peak expression at the indicated time point was calculated as a function of sex (*$P < .05$ and **$P < .01$). CRY, cryptochrome; EXP, experimental time point; F, female; M, male; MAX, maximum time point; ns, not significant.

healthy White males, with 2 subjects displaying an apparent phase delay (Wu et al, 2018). Given that our study used abdominal skin from primarily females and older individuals undergoing panniculectomies, it is perhaps not too surprising that these factors may lead to greater variability in clock gene expression. Nonetheless, clear rhythms and changes in gene expression could be observed for some of the genes in most of the subjects in our study. For example, in samples 2 and 12 from female donors aged 51 years and 35 years, respectively, we observed robust rhythms that closely modeled a sine wave for 3 of the 4 genes. Furthermore, for *NR1D2* and *CRY1* in sample 12, the expression profile exhibited patterns nearly identical to those seen in Wu et al (2018) (Figure 1d). Thus, abdominal skin explants may provide a reasonable

experimental model system for studying epidermal circadian rhythms. Panniculectomy and skin from other surgeries are routinely discarded from hospitals daily and are widely used by the field to study many aspects of skin biology. The large amount of skin makes it possible to examine many variables at 1 time. However, given that many aspects of skin biology are under circadian control and that circadian gene expression has yet to be monitored in skin ex vivo, our results provide a characterization of what circadian gene expression looks like in human skin epidermis ex vivo and may therefore be informative for studies of other skin parameters.

An additional difference noted in our study and those of Del Olmo et al (2022) and Wu et al (2018) was the more variable and distributed nature of the timing of peak gene expression throughout the day. This may reflect different chronotypes or sleep schedules of the donors in our study or some other factors associated with their health status (van der Merwe et al, 2022). Indeed, both studies recruited healthy subjects with a consistent sleep–wake cycle to sleep centers for their work. Moreover, the presumable need for surgical patients to fast prior to their surgery may have also impacted rhythmicity in the skin (Speksnijder et al, 2024). Furthermore, SNPs known to exist in these genes may have impacted their expression in the skin samples used in this study (Benna et al, 2017; Valladares et al, 2015). Finally, it is also possible that the ex vivo culture conditions used in this study contributed to differences ex vivo and in vivo, and thus, it will be important in future studies to determine how incubation medium, temperature, and other factors under experimental control impact rhythmic gene expression ex vivo. Thus, there are several potential caveats and limitations to our study that should be considered in the design of future and more comprehensive analyses of circadian gene expression in skin ex vivo, which include the use of complementary methods such as RNA sequencing and microarrays along with RT-qPCR validation to examine gene expression. Nonetheless, the results presented in this paper indicate that human skin explants may be a useful experimental model system to better understand circadian rhythms, their alteration in certain pathologies, their variations in individuals with more diverse demographics and health status, and their potential for pharmacological modulation (Chen et al, 2018; Ribeiro et al, 2021).

MATERIALS AND METHODS

Human skin samples

Experiments with human skin used discarded skin from routine panniculectomies of surgical patients providing written, informed consent at a local hospital, which was approved by the university institutional review board. Other than age and sex of the patients, no other identifying information about the patients or skin samples was provided. The skin donations were collected from the hospital at variable times between the hours of 10:30 AM and 4:30 PM within approximately 30 minutes after the surgery. Samples were then transported to the laboratory approximately 20 minutes away. Owing to the practicality of washing substantial amounts of human surgical skin and to mimic realistic, physiological conditions of human skin in a nonsterile environment, we used nonsterile tap water to wash the skin and remove excess blood. Sections (3 × 3 inches) of skin were then trimmed of excess fat and placed in 10-cm cell culture plates containing a small volume (approximately 5 ml) of basal DMEM (HyClone) in a standard laboratory benchtop water bath. The translucent water bath cover did not block the laboratory lights, and the plates containing the skin samples were set in the water bath at water level, which was set at 37 °C. At 6-hour intervals beginning at 6 PM, 8-mm punch biopsies were taken (at approximately 0.5 in distance from previous biopsies and the edge of the skin section) and stored in RNAlater at −20 °C until ready to be processed, which occurred days to weeks later.

RT-qPCR

Epidermis was separated from the dermis by briefly heating the biopsy in a water bath at 60–70 °C for 10–15 seconds and then placing in an ice bath for 10 seconds followed by scraping with a curette. Epidermal samples were homogenized on ice in TriZol using a BioMasher II pestle, extracted with phenol, and then purified using a RNeasy Plus Micro Kit (Qiagen). RNA was quantified on a NanoDrop One spectrophotometer, and then equal amounts of RNA were reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen). PCRs were set up using 2X TaqMan Fast Universal PCR Master Mix and TaqMan probes targeting *ARNTL* (Hs00154147), *NR1D2* (Hs00233309), *CRY1* (Hs00172734), *PER2* (Hs00256143), and *B2M* (Hs0187842). PCRs were run in at least triplicate reactions on an Azure Cielo 6 real-time PCR machine using an initial 3-minute melting step at 95 °C followed by 40 cycles of 95 °C for 10 seconds and 55 °C for 20 seconds. The $\Delta\Delta C_t$ method was used to determine fold changes in gene expression using *B2M* as a control gene for normalization. Relative gene expression over the time course was performed as previously described (Wu et al, 2018), in which the expression at each time point was normalized to the time point with maximum expression for each individual gene and skin sample.

Bioinformatic analyses

Transcriptomic datasets GSE205155 from Del Olmo et al (2022) and GSE112660 from Wu et al (2018) were downloaded from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/). Raw data were extracted and normalized from CEL files using the rate monotonic algorithm in the affy R package (Carvalho and Irizarry, 2010; Gautier et al, 2004) for data within the Wu et al (2018) study or normalized through the limma R package (Ritchie et al, 2015) for data within the Del Olmo et al (2022) study. Afterward, the gene names were assigned to the Affymetrix identifications using the HG-U219 Affymetrix Human Genome U219 Array in the Wu et al (2018)'s study or to the Agilent identifications using the Agilent-028004 SurePrint G3 Human GE 8x60K Microarray in the Del Olmo et al (2022)'s study. Both sets of gene name annotations can be found on Gene Expression Omnibus within each respective dataset. The time series datasets were analyzed with meta3d using the default settings in addition to cycMethodOne = ARS and period length being set to 24 for both minper and maxper in accordance with the protocol established in Wu et al (2018). Once both datasets were processed, circadian genes were extracted from each individual dataset using the semi join(dr) function in the dplyr R package and compiled to generate figures. Meta3d (Wu et al, 2016) was used to generate a *P*-value for each gene's expression pattern on the basis of how well the expression pattern seems to adhere to a temporal oscillation. For each gene in each dataset, we used the probe identification, which had the smallest *P*-value as determined by meta3d. After gene information was compiled, the data were normalized through the maximum (EXP/MAX) scaling method

(highest value set to 1). The normalized data were then plotted using GraphPad Prism (version 9.0) (GraphPad Software, 2023).

Mathematical and statistical analyses

Differences in RNA expression were evaluated using GraphPad Prism (version 9.0) and either an unpaired *t*-test or ordinary 1-way ANOVAs. To determine whether the gene expression profiles fit a sine wave function, we performed nonlinear regression analyses (sine wave curve fit with nonzero baseline and wavelength of at least 18 hours) using GraphPad Prism.

ETHICS STATEMENT

Experiments with human skin used discarded skin from routine panniculectomies of surgical patients who provided written, informed consent at Premier Health Miami Valley Hospital (Dayton, OH), which was approved by the Wright State University Institutional Review Board.

DATA AVAILABILITY STATEMENT

Gene expression datasets GSE205155 and GSE112660 were analyzed in this study and can be found at Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: MGK; Data Curation: WC; Formal Analysis: WC; Funding Acquisition: MGK; Investigation: WC, MGK; Methodology; WC, MGK; Project Administration: MGK; Supervision: MGK; Writing - Original Draft Preparation: WC, MGK; Writing - Review and Editing: WC, MGK

DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) OR LARGE LANGUAGE MODELS (LLMs)

The authors did not use AI/LLM in any part of the research process and/or manuscript preparation.

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